CHAPTER 4

Reversed-Phase High-Performance Liquid Chromatography

A Semipreparative Methodology

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1. Introduction

As solid-phase peptide synthesis techniques improved and the rate at which a peptide could be synthesized increased, purification was identified as the new bottleneck in the production of high-quality peptides. Purification took a giant leap forward with the introduction of reversed-phase high-performance liquid chromatography (RP-HPLC; *see* Chapter 3) to the synthetic laboratory. In fact, HPLC technology has been instrumental in the purification and characterization of most biologically active peptides and proteins *(1)*.

The difficulty level of the peptides attempted by solid-phase techniques has consistently increased, creating new separation problems. These problems include closely related species caused by side-chain modification, as well as deletion or addition sequences. Elimination of these impurities is crucial in order to assess the biological properties of a given compound accurately.

Additionally, since peptide drugs have now become a reality *(2–4)*, purification of intermediate and large quantities of these compounds has created a new demand—scale-up procedures from the analytical scale to semipreparative and ultimately the large commercial-scale purification. Analytical-level purifications are routinely performed on microbore and standard analytical columns. These separations generally separate from

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5 μg up to 1 mg of product depending on the complexity of the mixture. Semipreparative purifications are a broader, more ill-defined area since it can include samples from 5 mg up to 100 g. The macroscale purification is utilized at levels exceeding kilogram and multikilogram amounts.

This chapter will describe several different procedures that we have found to be extremely useful in separating fairly complex mixtures of crude products. Additionally, we have included examples of oxidatively folded types of molecules that present a purification nightmare to most researchers. The purpose of this chapter is to serve as a starting point for those with little or no experience in isolating peptides by semipreparative procedures.

2. Materials

2.1. Instruments and Columns

2.1.1. Preparative HPLC System

1. Waters, DELTA PREP 3000 Pump System (Max Flow Rate = 180 mL/min).
2. Waters Lambda Max (Model 481) LC spectrophotometer.
3. Waters 1000 PrepPak column Module (Standard radial psi = 700).
4. Chart recorder.

2.1.2. Analytical HPLC System

This consists of a Beckman System Gold: Pump Model 126, Detector Model 166.

2.1.3. HPLC Columns and Packings

Standard HPLC columns contain either spherical or natural (asymmetric) silica base derivatized with a polymeric carbon chain. The most common are octadecyl silica (C_{18}) linked columns and are most commonly utilized for small- to medium-sized peptides (5–50 residues). Larger and more hydrophobic peptides are more easily eluted from a C_4 column. C_8 columns are also commercially available, as well as columns with various ion-exchange substituents.

2.1.3.1. Prep System (2.0-g Load Capacity)

1. Waters PrepPak 500 Cartridge.
2. Delta Pak™ C_{18}, 300 Å, 15 μm column (47 × 300 mm).

2.1.3.2. Small-Scale Prep (Up to 100-mg Load Capacity)

This consists of a Vydac C_{18}, 300 Å, 15–20 μm (2.2 × 25 cm) column #218TP152022.

2.1.3.3. Analytical Scale (Fraction Analysis)

This consists of a Vydac, protein and peptide C_{18}, Å 5 mm, (0.46 × 25 cm) #218TP54.

2.2. Reagents

All reagents should be of the highest chromatographic quality to ensure accurate and reproducible results.

1. Acetonitrile (MeCN): Fisher Optima™ grade. A slightly lower grade may be substituted for large-prep runs because of repetitive washings, large volume, and high flow rate (4–6 L/run at 100 mL/min).
2. Trifluoroacetic acid (TFA): Aldrich 99+% (corrosive, toxic, hygroscopic). HPLC-grade TFA is essential to maintain chromatographic integrity.
3. Triethylamine (TEA): Fisher reagent grade (flammable, causes severe burns and irritation).
4. Phosphoric acid (H_3PO_4): Aldrich (85 wt% solution/H_2O) corrosive.
5. Sodium chloride (NaCl).

2.3. Mobile Phase

All mobile-phase formulations may be extrapolated to accommodate the specific purification scale. (In Section 3.2. and figure legends, A = Aqueous buffer; B = Organic modifier).

2.3.1. TFA System (4) *(pH = 2.6)*

1. 0.1% TFA/distilled H_2O (v/v) (HPLC-grade H_2O for analytical scale).
2. Acetonitrile (0.1% TFA for analytical scale).

2.3.2. Ammonium Acetate System

1. 0.05% Acetic acid/distilled H_2O (v/v); pH as desired (4–6) with NH_4OH.
2. Acetonitrile.

2.3.3. Phosphate System (5,6) *(TEAP) pH as Desired*

1. Triethylammonium phosphate (TEAP) 2.3 (pH = 2.3).
2. 0.0125% TEA/distilled H_2O (v/v), (adjust pH with H_3PO_4; *see* Note 1).
3. Acetonitrile (*see* Note 2).

2.3.4. Sodium Chloride System (7) *(pH = 2.5)*

1. 0.15*M* NaCl/distilled H_2O (pH to 2.5 with HCl).
2. Acetonitrile (*see* Note 2).

3. Methods

Purification of natural or synthetic peptides is not the result of a single scheme. It is, however, the qualitative consequence of the "synthesis-to-product" cycle. For that reason, each step is described below and illustrated in Fig. 1.

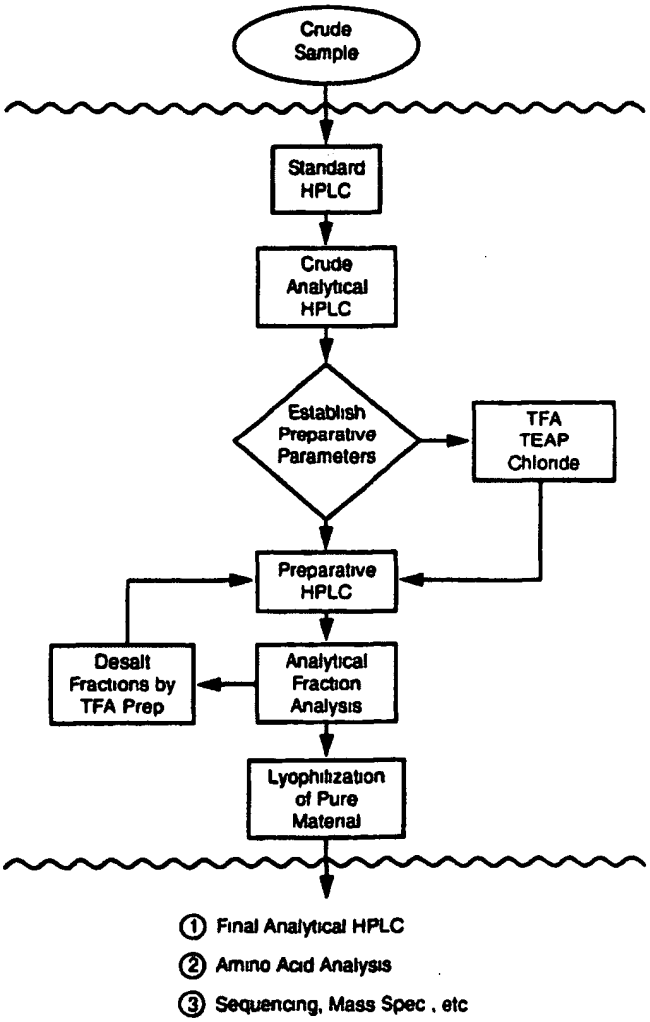

Fig. 1. Purification flowchart.

3.1. Sample Preparation

RP-HPLC analysis and purification require maximum solubility of the material in solvents most compatible with the instrument, while not hindering recovery of the peptide from the solvent. Crude products may be dissolved in a variety of aqueous-based solvent systems. Many of these may contain acetic acid, guanidine, urea, HCl, TFA, or MeOH in differing relative concentrations (*see* Note 3). These are not harmful to the

instrument, because the peptide remains hydrophobically bound to the column whereas the solubilizing agent is quickly eluted.

A number of prepurification steps may be performed to facilitate purification and increase column life. For example, crude peptides received directly from cleavage usually contain substantial amounts of residual scavenger or extracting solvents, such as ether or ethyl acetate. Lyophilization of this crude material followed by filtration will eliminate particulates and most residual scavengers, as well as volatile contaminants. Lyophilized solutions are generally water-based or easily evaporated. Furthermore, solutions containing water/acetic acid mixtures must be diluted to prevent thawing while lyophilizing. Samples that are dissolved in urea mixtures because of poor solubility should not be lyophilized, but loaded on the RP-HPLC column immediately. Alternatively, a guard column or an in-line precolumn filter may be attached to the system, but these may cause pressure discrepancies if not maintained properly, thus decreasing column efficiency or damaging the instrument.

Gradient formation must be determined from an analytical chromatogram prior to sample loading. Gradient formation, solvent selection, and detection are among the most important parameters, and will be addressed later in this discussion. Solubility is a key factor in liquid chromatography because the sample must be adsorbed to the column only to be desorbed later at the critical concentration by the organic modifier *(8)*. However, the solubilizing media must lie within the pH range of the column being used. Most reversed-phase columns are effective at acidic pH values and may be irreversibly destroyed by introduction of a solution of a higher pH value *(3)*. Finally, introduce a reasonable amount of material so as to maximize column and detector abilities. Overloading the system often greatly reduces the desired resolution and separation.

3.2. HPLC System Requirements

The HPLC system is usually prepared to the optimum parameters for the specific peptide prior to introducing the completed material (*see* Note 4).

3.2.1. Stationary Phase

One characteristic of an HPLC purification that the scientist can control is the stationary phase or "column packing." Most peptides and small proteins can be purified using reversed-phase C_4, C_8, or C_{18} columns. Columns linked with a C_{18} aliphatic chain are most commonly used for smaller and less hydrophobic peptides (<40 amino acid residues). Larger

and more hydrophobic peptides are more effectively purified by a C_4-substituted column *(9)*. C_8 packings work in much the same way as C_{18} and C_4, but requires intermediate values of solvent for elution of desired product. For most peptide applications, a pore size of 300 Å is recommended. However, in certain cases, such as extremely hydrophilic peptides or very difficult to resolve shoulders, a 100-Å pore size may be utilized to facilitate purifications by increasing surface interactions *(10)*.

3.2.2. HPLC Mobile Phase

The mobile phase of the HPLC system is the most important variable with respect to determining component elution. It is a combination of varying percentages of aqueous and organic solvents, utilizing a variety of gradient conditions, such as linear, hyperbolic, step, and isocratic elution. Selection of solvents and gradient conditions will determine the behavior of the target molecule.

Two of the most useful and convenient systems are those involving: (TFA system *[5,6]*) acetonitrile vs distilled H_2O (0.1% TFA) and a more ion-pairing system involving triethylammonium phosphate (TEAP *[7]*): acetonitrile vs distilled H_2O (buffered to a specific pH with TEA and H_3PO_4). Acetonitrile is preferred by this author because of the ease with which it can be removed by lyophilization to yield a "fluffy" peptide, as well as the excellent absorbance properties at wavelengths at which the peptide absorbs.

The TFA system is ideal for cases where the product peak is a large percentage of the total crude cleavage mixture or there are no closely eluting hydrophilic or hydrophobic contaminants, known as "shoulders," on the desired peak. A simple one-step HPLC purification is easily accomplished followed by lyophilization of the desired fractions collected.

However, in such cases where the synthesis or crude material is heavily contaminated, the use of one or more of the TEAP systems followed by a TFA desalting run may be necessary. This TEAP strategy offers enhanced purification capabilities in most cases *(6)*, but requires greater time and effort commitment, and is therefore only used when necessary. Generally, TEAP 2.3 is used as the initial purification, with consequent TEAP runs of increasing pH as needed (up to pH = 7.0 for column stability). When an appropriate level of purity has been achieved, the phosphate salt is removed by diluting with H_2O (2–3×) and reloading the pure material. A shorter gradient may be utilized to desalt the material on the

TFA system because of the higher degree of purity. Also, lyophilization of TEAP fractions will result in a harmful phosphoric acid syrup—hence the need for subsequent desalting.

3.2.3. System Operation

Optimal separation and resolution are achieved by gradient determination specific to each peptide and are directly comparable to the analytical profile. Therefore, the analytical profile is the major reference point for parameter determination by giving sample purity and identification of contaminant shoulders and approximate organic solvent concentration needed for elution (*see* Note 5). Consequently, one may predict a suitable solvent system as well as appropriate gradient conditions. **Note:** The TEAP system is used for situations where closely eluting shoulders appear, and material tends to elute 5–8% (B) (organic) earlier in TEAP than in an identical TFA system *(6)*. Retention time, resolution, and separation are affected slightly by the amount to be purified. Quite often, results improve at lower levels (200–700 mg of most peptides at the preparative scale). Most peptides act favorably in TEAP systems. However, there are exceptions that are difficult to recover successfully (*see* Notes 6 and 7).

3.2.3.1 System Start-Up and Gradient Determination

The standard RP-HPLC system is stored at 100% of the organic modifier for overnight and multiday periods to prevent microbacterial accumulation in aqueous solvents. The system must be re-equilibrated to the initial starting conditions prior to sample loading. We generally employ a reverse gradient to accomplish this procedure. A reverse gradient is effected by accomplishing a rate of change, from 100% of the organic modifier to 0%, resulting in maximal cleansing of the RP-HPLC column in a reasonable amount of time. A time of 20–30 min is generally sufficient, followed by approx 10 min isocratically at 0% B, or the initial starting conditions, to equilibrate the system fully. Three to five column volumes of aqueous solvent are often a sufficient volume for this purpose.

The RP-HPLC system must be checked for correct operation parameters. If the system is equipped with a radial compression chamber, the radial pressure must be checked for a steady and suitable operating pressure. The Waters Delta-Prep 3000 operates optimally at 650–700 psi. It is imperative to maintain a steady system pressure throughout the HPLC run. Therefore all leaks and excessive backpressures must be eliminated.

Clogged precolumn filters as well as filter paper applied directly to the RP-HPLC column are most commonly found to produce increased pressure readings. These should be checked and replaced regularly. Pressure variations are also a consequence of irregular flow patterns. Flow rates must be examined routinely and at different flow rate values, thereby revealing any pump system or general flow discrepancies. Irregular flow and major pressure fluctuations may also result from worn or damaged check valves.

Detection parameters are vital to each RP-HPLC run and should be adjusted accordingly prior to each sample loading. The pertinent variables to be addressed are the detection wavelength and the range of the detector.

Wavelength selection is commonly set at approx 230 nm for the semipreparative scale. The peptidyl backbone is easily detected at 220 nm. However, limiting factors, such as larger sample size or smaller flow cell dimensions, indicate a need for a slightly higher wavelength setting to reduce sensitivity of the instrument.

The range function can be viewed as a "window," and operates in a similar fashion. The range employs aufs units (absorbance units full scale) and translates absorbance data to the chart recorder. The range scale commonly exhibits settings as 0.001, 0.01, 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 increasing in magnitude. Increasing values expands the range and therefore decreases sensitivity. Likewise, decreasing range values results in increased sensitivity of detection, allowing one to see minor components more easily. Sample sizes of 100–500 mg can be effectively run at a range of 0.2–0.5 aufs. The range should be increased according to the number of aromatic residues or fluorescent groups contained in the peptide. Additionally, alternative wavelengths can be employed, such as 254, 275, 280, or others depending on the wavelength that allows one to observe only components containing aromatic residues. With these wavelengths, the range must be decreased because of the lower absorbance value at these wavelengths.

Following a check of all parameters, a gradient is constructed, and the sample loaded at either 0% of the organic modifier or the initial conditions of the gradient. Generally, this gradient is constructed to accomplish a specific rate of change of organic modifier, relative to an aqueous cosolvent. This gradient must result in a rate of change sufficient to achieve maximal separation of impurities from the desired product (*see*

Note 8). As a general rule, the gradient is designed so the elution percentage of the organic modifier is the midpoint of the gradient and the sample is loaded at the initial gradient conditions. Our laboratory typically loads samples at 0% organic modifier to ensure sample binding to the column matrix. Consequently, a peptide eluting from the column at 30% of the organic modifier analytically will be run on a gradient of approx 15–35% at the semipreparative level, with the sample being loaded at 0–15% organic concentration.

For efficiency purposes, the gradient should be designed for a run time of approx 1 h. This will allow for a maximum number of runs and column washes in a working period, while maintaining efficient separations. However, maintaining the percentage change in organic modifier while increasing run time (10–15 min) may also increase separation.

Equilibration to the initial conditions, following sample adsorption to the column matrix, allows elution of solubilizing agents, such as acetic acid and urea, as well as early eluting contaminants. The gradient program is then initiated, and the observed eluting peaks collected manually or automatically with a fraction collector into appropriately sized containers, such test tubes or flasks. Optimal separation of even moderately impure samples is achieved by limiting the size (volume) of the individual fraction collected. In our laboratory, we have employed the following standard parameters: a flow rate of 100 mL/min, gradient 20% change in organic modifier in 1 h, and the fractionation method by manual collection in 1.6 × 25 cm test tubes. Also, more complex mixtures may be more easily purified by collecting even smaller volumes or "half-fractions."

Alternatively, most instruments offer a "pause" feature that allows a gradient to be held at any point during the run. Use of this feature is helpful for separating closely eluting contaminants by effecting an isocratic procedure (a constant unchanging concentration of organic modifier) within the gradient, wherein the gradient is operated up to a point near the peptide eluting concentration. The gradient is halted at the particular organic percentage, allowing a gradual separation of product from contaminants.

The practice of isocratic elution may be taken a degree further by employing an entire isocratic RP-HPLC run at a constant organic modifier concentration over a predetermined time parameter. Care must be taken to ensure efficient binding of the sample to the column's stationary matrix. Consequently, the product gradually separates away from contaminants

Table 1
Purification Yields for Representative Peptides

Peptide	Crude start amt.	HPLC	Final yield
CRF	500 mg	① 0.15*M* NaCl	100 mg
	100 mg	② TEAP 4.7	(semipure)
		③ TFA desalt	24 mg
GRF	4 × 500 mg	① TEAP 2.25	1.161g
		② TEAP 6.8	
		③ TFA desalt	
Echistatin	1.8 g[a]	① TEAP 2.3	91 mg
		② TFA desalt	
Charybdotoxin	1 8 g[a]	① Drop sample pH to 2.5	80 mg
		② TEAP 2.3	
		③ TFA desalt	

[a]These numbers represent an approximation of theoretical weight based on the final resin weight. These samples were oxidized directly without lyophilization following cleavage.

at the midpoint of the run. Generally, the concentration is kept constant over a time period of 30–60 min. Also, mixtures of different organic modifiers, such as isopropanol:acetonitrile, may be utilized in an isocratic procedure to enhance separation parameters further.

Following a successful prep run, one must analyze the fractions that were collected. Fraction analysis is of vital importance and, therefore, should be analyzed by the most rigorous methods available (*see* Note 5). Analytical columns and reagents should be of the highest grade available and the gradient formation equally as rigorous. A smaller percent change in organic modifier and a lower flow rate (~1 mL/min) often provide the desired results.

Finally, the RP-HPLC semipreparative column is washed vs the organic solvent and equilibrated prior to the next run. Multiple gradient washings at lesser time intervals (0–100% B in 10 min × 3) often are more effective than one longer wash (0–100% B in 30 min × 1). Following the column wash, the system is equilibrated to 0% B or the subsequent initial conditions. For storage purposes, the system should remain equilibrated in 100% of the organic solvent.

3.3. Purification Examples

3.3.1. Growth Hormone-Releasing Factor (GRF) (11)

N-Acetyl-Tyr_1-D-Arg_2-GRF(1-29)AMIDE(human)(GRF Antag) (Table 1): The peptide was dissolved in 20% AcOH. The GRF antagonist

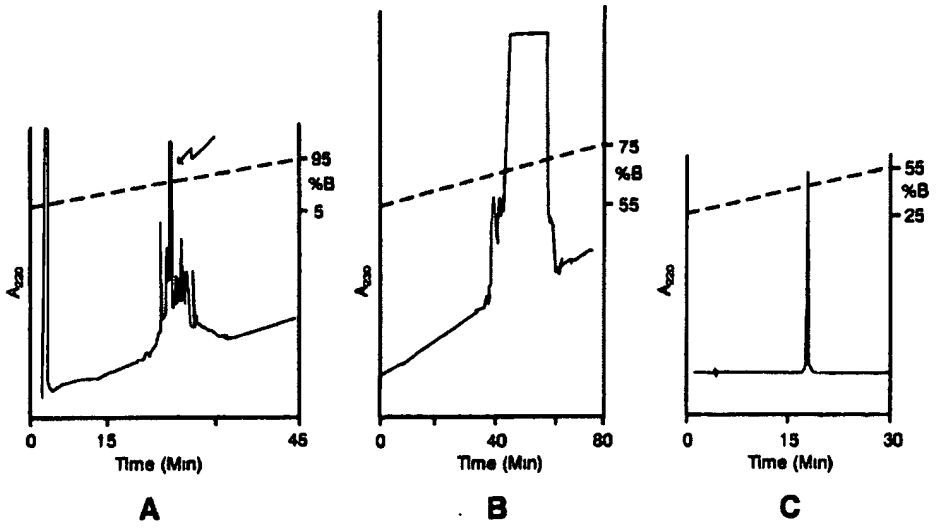

Fig. 2. GRF antagonist: (A) Crude mixture analysis: analytical; 5–(45 min)–95% B; flow rate = 1.5 mL/min; chart speed = 0.5 cm/min; range = 0.2 aufs. (B) Preparative HPLC: 55–(80 min)–75% B; flow rate = 100 mL/min; chart speed = 0.25 cm/min; atten. = 0.5 aufs. (C) Final pure analytical HPLC: 25–(30 min)–55% B; flow rate = 1.0 mL/min; chart speed = 0.5 cm/min.

was purified by TEAP 2.3 followed by TEAP 6.8 to eliminate closely eluting contaminants (Fig. 2A), and subsequently desalted on the TFA system prior to lyophilization. This procedure resulted in a product with a purity level of >98%. However, a later purification run demonstrated the product to oxidize partially at a Met during the higher pH procedures and without resolving the contaminants at lower pH runs. This peptide modification was eliminated and the contaminants separated by employing a unique solvent system (Fig. 2B). This specific system consisted of an aqueous solution (A) of 0.15*M* NaCl (pH 2.4 with HCl) and the organic modifier (B) of 10% MeCN in MeOH. The resulting product was desalted using a TFA prep, active run prior to lyophilization. Purity was determined to be >98% (Fig. 2C).

3.3.2. Corticotropin-Releasing Factor (CRF) Ovine (12)

CRF ovine (Table 1) was solubilized in 50% AcOH for purification. The crude RP-HPLC profile showed a broadening of the target peak (Fig. 3A) as a result of closely eluting contaminants. The TEAP system

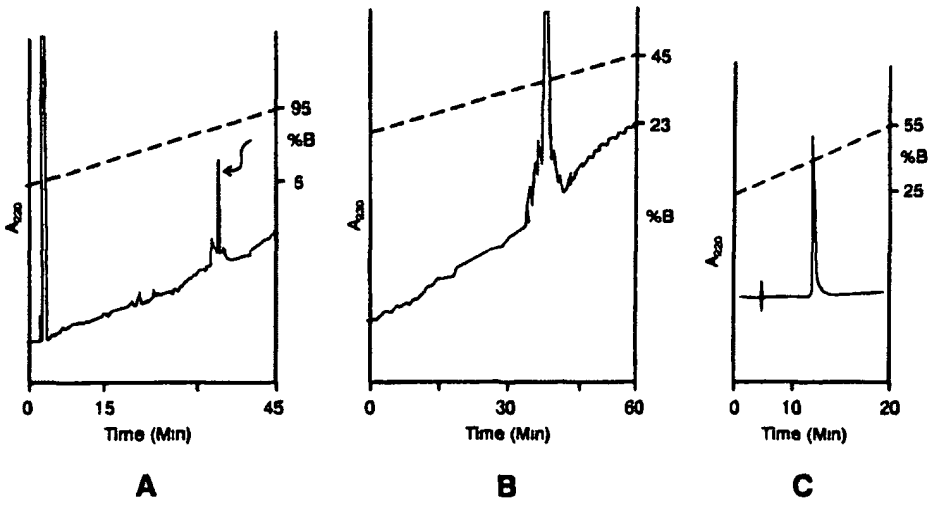

Fig. 3. CRF ovine: (A) crude mixture after lyophilization and filtration; linear gradient of 5–(45 min)–95% B; flow rate = 1.5 mL/min, chart speed = 0.5 cm/min; range = 0.2 aufs. (B) Preparative HPLC TEAP 2.3: linear gradient of 23–(60 min)–45% B; flow = 100 mL/min; chart speed = 0.25 cm/min; atten. = 0.5 aufs. (C) Final pure analytical HPLC: gradient = 25–(20 min)–55% B; flow 1.0 mL/min; tailing is seen routinely with CRF ovine.

was employed because of its excellent separation abilities under these circumstances (Fig. 3B). The final pure product (Fig. 3C) was obtained following a TFA desalt RP-HPLC and lyophilization.

3.3.3. Echistatin

Echistatin (Table 1) is a 49 amino acid polypeptide from the venom of the saw-scaled viper, *Echis carinatus*, and contains four disulfide bonds *(13,14)*. The free peptide was air-oxidized, and the crude RP-HPLC showed two peaks of similar intensity. The latter peak is the target peak, the earlier eluting peak represented an oxidation of methionine-to-methionine sulfoxide (Fig. 4A). Purification by TEAP 6.0 resulted in the most efficient separation of the two peaks at the semipreparative level (Fig. 4B). The relevant fractions were desalted by TFA RP-HPLC and lyophilized yielding a product of >98% purity (Fig. 4C).

3.3.4. Charybdotoxin

Charybdotoxin (Table 1) is a 37 amino acid peptide found in *Leiurus quinquestriatus*, scorpion venom, and contains three disulfide bonds *(15)*.

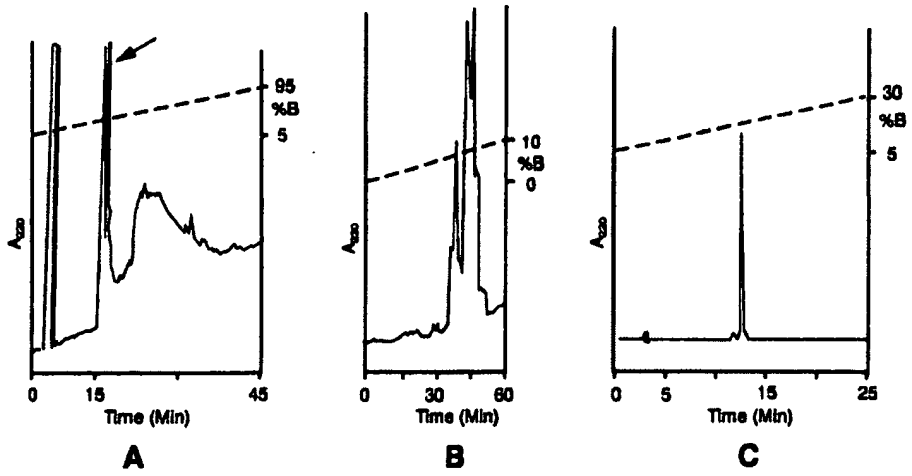

Fig. 4. Echistatin: (A) Crude mixture after disulfide bond formation; gradient = 5–(45 min)–95% B; flow = 1.5 mL/min; atten. = 0.2 aufs. (B) Preparative HPLC: gradient = 0–(60 min)–10% B; flow = 100 mL/min; atten. = 0.1 aufs. (C) Final pure analytical HPLC: gradient = 5–(25 min)–30% B; flow = 1.0 mL/min.

Shown in Fig. 5A is the fully reduced crude peptide amidst a mountain of peaks. On air oxidation, the biologically active molecule migrated to a retention time preceding the bolus of contaminant peaks (Fig. 5B). The pH of the solution was dropped to 2.5 prior to loading onto the RP-HPLC column. In order to ensure sufficient purity, the material was eluted from the column using the TEAP 2.3 system (Fig. 5C). The resulting fractions were desalted by the TFA RP-HPLC system and lyophilized to yield a product of >98% purity (Fig. 5D).

4. Notes

1. The TEAP system may be adjusted as desired in order to maximize the individual purification by varying the pH values between 2.3 and 6.8.
2. It is advisable to store the HPLC system in 100% acetonitrile to avoid halide corrosion of the metal parts and prevent microbacterial accumulation in aqueous solvents. The system must be washed following purifications involving halides.
3. Lyophilization of peptides solubilized in urea or guanidine is not recommended. A desalting run using the TFA system is recommended instead. This will produce a more realistic RP-HPLC profile and, combined with sample filtration, will maintain column integrity.

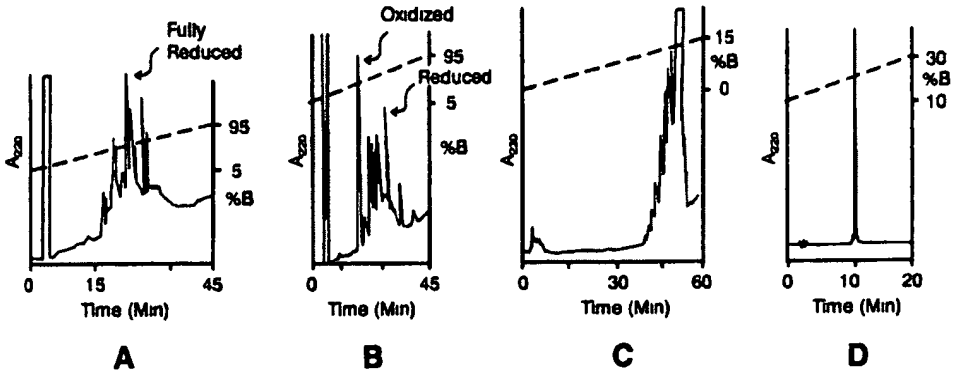

Fig. 5. Charybdotoxin: (A) crude sample before air oxidation; gradient = 5–(45 min)–95% B; atten. = 0.2 aufs.; flow = 1.5 mL/min; inj. vol. = 250 mL of 4-L solution. (B) Crude sample following disulfide bond formation; HPLC conditions same as above. (C) Preparative HPLC, TEAP 2.3: linear gradient = 0–(60 min)–15% B; atten. = 0.2 aufs.; flow = 100 mL/min; (D) Final pure analytical HPLC: gradient = 10–(20 min)–30% B; flow = 1.0 mL/min.

4. The smallest change in one of the purification parameters may greatly increase the difficulty of the immediate operation. For example, a minor change in system, such as fluctuation of room temperature, may result in differences in elution time as well as contaminant separation, thus causing irreproducible results.
5. Another common variable is the pre- or postpurification oxidation of susceptible amino acids (Cys, Met). This problem, although inconvenient, is a preventable problem. Extreme hydrophilicity or hydrophobicity of a peptide can create an ineffective purification owing to premature or incomplete elution from the column matrix. Since most alkyl chain-linked columns have an effective sample load range (100 mg–2.0 g) and pH (2–7.5) *(3)*, these parameters may be varied to affect the peptide purification. The following examples are of unique situations encountered in our lab as well as possible solutions.
6. Each HPLC run results in partial sample loss owing to nonspecific adsorption to the column matrix. Therefore, minimizing the number of runs will give a greater yield in recovered product.
7. Hydrophilic peptides that do not adsorb well to the column matrix may be more successfully purified using a smaller pore size particle (100 Å), thus promoting increased surface interaction *(10)*.
8. Never discard eluent prematurely! If sample is not observed eluting during run, one may check the loading wash as well as the 100% acetonitrile wash to find the product. Absorbance detection may misrepresent actual product yield.

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